Volume 307, number 1, 71–75 FEBS 11209 July 1992

Minireview

Monoclonal antibody engineering in plants

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Received 11 May 1992

Techniques for plant transformation have been developed to such an extent that a number of foreign genes are currently being introduced into transgenic plants. Tobacco plants that produce monoclonal antibodies are of interest, because in addition to synthesis of two gene products (i.e. the heavy and light chains), the two polypeptides need to be assembled correctly, in order to result in a functional antibody. The studies on a catalytic antibody suggest that this is the case, and that the antibody functions identically to the native murine-derived antibody. The only difference observed was in the glycosylation of the heavy chain. Further transgenic plants are being generated to produce monoclonal antibodies that may be used therapeutically (and are therefore required in large quantities), or to provide disease resistance in plants. In addition, the ability of plants to assemble antibody complexes is being investigated further, to study the possibility of generating secretory IgA, which consists of heavy and light chains as well as two additional polypeptide units.

Monoclonal antibody; Transgenic plant; Immunoglobulin heavy chain; Immunoglobulin light chain; Immunoglobulin assembly; Passive immunisation

1. INTRODUCTION

A variety of bioactive compounds have been stably introduced into plants using genetic engineering techniques [1–12]. These compounds, by and large, are derived from mammalian, bacterial, or viral genes which encode well-characterized protein products. In some instances other bioactive plant products have been introduced into a new plant environment to obtain a useful trait. There are three characteristics of plant cells which make them valuable tools for the synthesis and analysis of bioactive compounds. Firstly, many plant cells can be propagated either as clumps of cells on agar plates (callus), as liquid suspension cultures, or as protoplasts from which the cell walls have been removed [13–15]. Secondly, whole plants can be regenerated from callus cultures or from protoplasts [13,16]. Thirdly, sexual crossing of transgenic plants can be used to introduce additional genes into the progeny. Thus the genes required for complex oligomeric compounds can be accumulated by a series of crosses.

Expression of monoclonal antibodies in plants is of particular interest, because it requires the expression of two genes, synthesis of two proteins and subsequently, the correct assembly of the tetrameric protein to result in functional antibody. The fidelity of such assembly is ensured in cells which normally synthesize immunoglobulins because of the recognition of processing and signal sequences in the immunoglobulin genes and because of specialized cellular machinery to accommodate these functions. However, it was not known whether plant cells could fulfill these requirements.

The initial studies of antibodies in plant focussed on the IgG class of antibodies, although as discussed later, we are now also investigating the production of multimeric forms of immunoglobulin. This paper will review the properties of antibodies produced in plants and discuss the potential use of this technology for the production of forms of antibody which have not been possible by conventional techniques.

2. PLANT TRANSFORMATION AND ANTIBODY SYNTHESIS

The most commonly used method for DNA mediated transformation of plants, is to employ *Agrobacterium tumefaciens* as the 'delivery vehicle' for introduction of recombinant vectors to the plant cell nucleus [17–19]. Not all plants are amenable to the manipulations required for the stable introduction of foreign DNA. Tobacco is the most commonly used plant, since it is easily transformed and regenerated. However, a tremendous effort is being made in various laboratories to perfect transformation techniques for common crop plants such as corn, soybean, alfalfa and rice.

As plant expression vectors are generally large already [28], and contain only one promoter and one polylinker region, it is probably wise to express only one immunoglobulin in each vector and to transform sepa-

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rate plants with individual heavy and light chain-expressing vectors. As described below, plants expressing functional antibodies are produced in the progeny of a cross pollination between the individual heavy or light chain containing plants.

A murine IgG1 antibody, that recognises a synthetic phosphonate ester and catalyses the hydrolysis of certain carboxylic esters [20] has been studied. γ and κ chain cDNAs were cloned individually into the plant expression vector pMON 530 [21]. This vector contains plant selectable markers, promoters, *E. coli* and *Agrobacterium* origins of replication and a mouse immunoglobulin signal sequence upstream from the PCR insert. Transgenic plants were regenerated using these constructs [22].

The levels of expression of γ or κ chain in transformed plants are shown in Table IA. Overall, γ chain expression was higher than κ chain expression. Transgenic plants containing γ or κ chains were then crossed to produce progeny expressing both chains. The accumulation levels of γ and κ chains in the F_1 plants, γL(κL) and κL(γL) respectively, are also shown in Table IA. γ and κ chains were expressed in equivalent amounts — 3330 ng/mg and 3700 ng/mg, respectively, and at much higher levels compared with the previous generation of plants that only had one insert.

The distribution of antibody chain gene expression in the F_1 progeny is shown in Table IB. In a cross between γ and κ chain plants, 11/18 of the offspring expressed γ and κ chains together. Furthermore, assembly was observed in the majority of these plants (95%). A proportion expressed only one chain and 4 plants expressed neither. That the F_1 plants contained assembled functional antibody, was determined by the following criteria: (1) Western blots of plant extracts; (2) ELISA assays in which antibody in plant extracts was captured with goat anti-mouse γ chain and detected with HRPO labelled goat anti-mouse κ chain; (3) ELISA assays using the native antigen in which the affinity of γ–κ complexes for antigen was identical to that of the hybridoma-derived antibody.

The functional activity of the plant-derived antibody was compared with that of the ascites derived antibody. Antigen specificity was confirmed by inhibition of binding by free antigen, in which the half minimal inhibition was about 10 mM for both antibodies. The catalytic activities of both antibodies differed by less than an order of magnitude for each parameter examined (Table II). These measurements are within the observed range for different batches of the ascites produced antibody (Janda, unpublished data).

A surprisingly high level of accumulation of functional antibody was observed amounting to greater than 1% of total extractable protein. Other antibodies, which have subsequently been expressed in tobacco using the same strategy have resulted in similar levels of accumulation.

Table I

Expression and assembly of immunoglobulin γ and κ chains in tobacco

A. Accumulation of γ or κ chains in transformed plants. Results are expressed as mean ± S.D. ng/mg total protein, estimated by ELISA assay.

γ	γ (κ)[a]	κ	κ (γ)[a]
1,412 ± 270 (2,400)	3,330 ± 2,000 (12,800)	56 ± 5 (80)	3,700 ± 2,300 (12,800)

[a] γ(κ) refers to γ chains in a plant that also expresses κ chains, and vice versa. Numbers in parentheses are values for plants with the highest levels of accumulation.

B. Distribution and assembly in crosses. Results are expressed as the number of plants expressing γ or κ chains among the progeny of a sexual cross.

	γ only	κ only	γ κ	null
$\kappa \times \gamma$	3	10	11 (95 ± 16% assembly)	4

3. CHARACTERISTICS OF THE FC REGION

Purified antibody was prepared using Sephacryl FPLC and Protein A-Sepharose. The quantitative retention on Protein A-Sepharose indicates that the region between the CH2 and CH3 domains that is recognised by protein A was intact.

Evidence that the antibody had been proteolytically processed in the lumen of the ER was provided by the N-terminal amino acid sequence. Heavy chain from either plant antibody or mouse derived 6D4 was intractable to sequencing, indicating a blocked N terminus. The light chain N-terminal sequence was Asp-Val-Val-Leu for both plant and mouse antibody. This demonstrates appropriate proteolytic processing of the mouse signal sequence by the plant ER.

Glycosylation of the antibody heavy chain was investigated by lectin binding analysis [23,24]. Both plant and ascites γ chain were bound by Concanavalin A (specific for mannose and glucose), but ascites γ chain was also recognised by lectins from *Ricinus communis* (specific for terminal galactose and *N*-acetylgalactosamine) and wheat germ agglutinin (*N*-acetylglucosamine dimers, terminal sialic acid); [25]. This suggests a different com-

Table II

Catalytic activity of the 6D4 antibody produced in tobacco

Source	Tobacco	Ascites
K_m (M)	1.41×10^{-6}	9.8×10^{-6}
V_{max} (M sec^{-1})	0.057×10^{-8}	0.31×10^{-8}
K_i (M)	0.47×10^{-6} (competitive)	1.06×10^{-6} (competitive)
k_{cat} (s^{-1})	0.008	0.025

position of terminal residues on the plant glycan and is consistent with the absence of NANA in plants. The lectins from *Datura stromonium* (*N*-acetyl glucosamine oligomers, *N*-acetyl lactosamine) and *Phaseolus vulgaris* (galactose-β1,4-*N*-acetyl glucosamine-β1,2-mannose) did not bind to either plant or ascites-derived γ chain.

Both glycans were resistant to endoglycosidase H, which is a characteristic of complex carbohydrates processed in the Golgi apparatus and indicates that the transgenic antibody is processed in a similar fashion to complex mammalian glycoproteins. In addition, both glycans have approximately the same affinity for Con A since they were not distinguishable by competition with α-methyl mannoside. This type of assay has previously been used to distinguish a variety of plant glycans with respect to their affinity for lectin [26].

4. FURTHER STRATEGIES FOR EXPRESSION OF ANTIBODIES IN PLANTS

Vectors encoding Ig heavy and light chains can be introduced into tobacco protoplasts by electroporation or by using polyethylene glycol as a facilitator [27]. Transient expression results in the synthesis, assembly and secretion of functional antibodies. This system is more rapid than plant transformation and regeneration and has been used to investigate a variety of vector constructs based on differences in the type and arrangement of promoters.

In general, we found that different antibody structures can be produced from plant cells by a number of promoter arrangements in the vector. Two types of constructs were made to measure the effects of variations in levels of transcription on the accumulation of secreted antibody. In the first construct, the heavy or light chain expression cassette containing the 35S promoter, cDNA and 3′ nos region in pMON530 [21] were introduced into the vector pKYLX71 [29] at the *Hin*dIII site. This resulted in a vector with two 35S promoters upstream from the cDNA. The expression of secreted antibody from protoplasts electroporated with these vectors was at least 10-fold more efficient than constructs employing a single 35S promoter.

In a second construct, the 35S promoter was substituted by the HSP 70 heat-shock inducible promoter from soybean [28]. Although heat-shock induction of antibody production was not achieved in protoplasts, plants that were regenerated after leaf-disc transformation using the same vectors, expressed γ and κ chains only after exposure to 37° C for one hour. Some of the progeny from a cross between γ and κ expressing plants expressed functional antibody, but only if both parent plants had been transformed with the HSP 70 construct. No antibody was expressed if one parental plant contained the 35S promoter and the other expressed the HSP 70 promoter.

The results from analysis of regenerated plants expressing heavy and light chain constructs suggested that the native signal sequence on the mouse transcript contributes significantly to the accumulation of heavy or light chains [22]. We have also found that virtually any signal sequence can be employed, including other mouse signal peptides as well as pro-sequences derived from microorganisms. cDNAs with no signal sequence have also been introduced into protoplasts by electroporation [30,31]. Expression of individual chains was barely detectible and when both chains were co-expressed, there was no assembly of γ–κ complexes. This effect is presumably due to the sequestering of the chains in the endomembrane system and their subsequent secretion when leader sequences are present. Our results suggest that plants must contain an assembly and processing apparatus which can recognize mouse immunoglobulins. The efficiency of assembly in plants was surprising since there was a large disparity in parental levels of expression of indidivual γ or κ chains. After crossing, one of the antibody producing progeny contained 5-fold more γ and 160-fold more κ chain than the respective parental plants. This suggests that the assembled antibody is more stable in plants than the individual heavy or light chains. It is apparent that cognate mechanisms function in plants to coordinate the assembly of oligomers, to direct *N*-glycosylation and to effect processing in the Golgi apparatus, to recognize and to process immunoglobulins efficiently. In mammalian cells, assembly of immunoglobulin chains is thought to occur via a native component of the endoplasmic reticulum (BiP), which is involved in the post-translational processing of heavy chains [32] and a BiP-like protein has recently been characterized in plants [33,34].

In order to express both γ and κ chains in the initial transformant, promoter arrangements whereby γ and κ cDNAs are encoded in the same vector have been tested. A vector, pHi202 [22] was used which contains the leader and γ chain insert. In addition, a *Hin*dIII fragment containing an additional 35S promoter [21], the light chain cDNA, and the nopaline synthase gene 3′end were introduced. The two orientations of the double expression vector were then introduced into protoplasts. Only the 'head-to-tail' orientation resulted in expression of assembled antibody. The efficiency of expression (as measured by the ng of antibody produced from 10^6 cells electroporated with 20 mg DNA) was significantly higher than electroporation using two vectors.

Most of the characterisation of antibodies from plants performed to date, has been on antibodies which have been targeted for secretion through the plasma membrane [22]. Alternative techniques for the efficient production of intracellular antibodies have yet to be tested. One possibility is the single chain antibody construct [35–37] in which endomembrane associated assembly of heavy and light chains is replaced by refolding

of variable regions joined by a peptide linker. This obviously requires a considerable amount of molecular engineering, but if successful, offers a realistic approach to introducing antigen binding to the plant cytosol or other compartments.

5. APPLICATIONS OF PLANT DERIVED MONOCLONAL ANTIBODIES

Although transformation of plants is a lengthy process, there are several advantages over other expression systems. The prospect of harvesting monoclonal antibody on an agricultural scale would mean that antibodies would be available extremely cheaply in almost limitless amounts. One of the most attractive potential uses for large quantities of antibody is in passive immunisation. Monoclonal antibodies have been used in vivo for many human diseases with varying degrees of success and recently we have been interested in the possibility of orally delivered MAb. If the right transgenic plants were used, the antibody might be administered directly, without the need for extraction or purification. Topically applied MAb has been used successfully to prevent oral disease in both sub-human primates [38] and humans [39]. Transgenic plants that produce the MAb Guy's 13 that was used in the human studies, are currently being constructed.

Transgenic plants offer a number of advantages with respect to vaccine storage and distribution. Plant genetic material is readily stored in seeds, which are extremely stable and require little or no maintenance. Unlike the considerably more stringent requirements of bacterial or mammalian cells, seeds have an almost unlimited shelf life in ambient conditions. Immortalisation of the plant line therefore is extremely simple, furthermore, mature plants can be self fertilised to produce identical offspring, a technique that has been established in plant breeding programmes for many years. As the number of transformable plant species increases, to include several of the major food crops such as rice, potato, cassava and peppers, the possibility arises of delivering antibody vaccines on a global scale, using plants that are indigenous to particular regions. In this way, vaccines could be administered cheaply to third world countries by utilising the existing agricultural infrastructure.

In addition to the economic advantages of using plant derived antibodies, the ease with which genetic material can be exchanged by cross fertilisation, may facilitate the construction of multimeric forms of antibody. The ability of plant cells to assembly heavy and light antibody chains correctly, has been demonstrated [22]. The possibility of constructing antibodies that require more than two polypeptide chains is being explored, and the secretory form of IgA (sIgA) is of particular interest. sIgA is usually present at mucous membranes as a dimer, consisting of two monomeric IgA molecules joined by a small polypeptide (J chain), and complexed with a larger polypeptide (the secretory component) [40]. Although J chain is synthesised by the plasma cells that secrete the IgA, secretory component is expressed by epithelial cells that are present in the secretory glands. Because of this, it has not been possible to generate monoclonal sIgA using standard hybridoma techniques. However, the genes that encode the four component chains of sIgA could be incorporated into one plant by cross breeding, although it remains to be determined whether correct assembly of antibody woud occur.

As the majority of infectious agents are initially encountered at mucosal sites of the body, topical passive immunisation with sIgA may be an effective measure against many diseases. This approach would overcome the difficulties previously encountered with regard to inducing high and long lasting titres of specific antibody [41], as well as avoiding the possibility of developing oral tolerance.

Other multimeric antibody molecules could also be constructed in plants. Thus, the product of two transgenic plants, each producing different antibodies, would be a bifunctional antibody and in theory, any combination of heavy and light chains could be made. In addition, we are alsc investigating the possibility of engineering a secretory form of IgG, which would be multivalent and might be more stable in the mucosal environment, yet still retain the functions of the IgG Fc region.

Antibodies have previously always been considered as part of mammalian defence systems. The possibility now arises of utilising pathogen-specific antibodies to provide disease resistance in plants. The effector mechanisms in plants may be limited to agglutination, enzyme inhibition and blocking of essential antigenic epitopes. However, it may also be possible to engineer antibodies that could take advantage of plant mechanisms for disease resistance. For example, plant toxins could be targetted to invading organisms by conjugation with specific antibody. Alternatively, genes for pesticides, such as the δ-endotoxin of *Bacillus thuringiensis*, could be engineered within those encoding the antibody, in order to target these pesticides to the plant pathogen.

6. SUMMARY

Of the variety of compounds expressed in transgenic plants, antibodies probably offer the widest range of applications. The antibodies appear to possess all of the functional characteristics of those derived from hybridoma cells, although further study will be required to determine the effect of the difference in heavy chain glycosylation. More work will also be directed toward the assembly, accumulation, stability and secretion of plant antibodies. Methods for achieving high level expression of foreign gene products consistently in the new plant environment have not been established yet.

However, there is a range of options potentially available and temporally controlled (developmental or inducible), tissue-specific, if cell-specific expression of the gene may lead to the desired result. In the case of tobacco, the accumulation of antibodies throughout the plant accounted for more than 1% of the total extractable protein. Conceivably, with some manipulation of the transcription rates, the accumulation of an antibody either in whole plants or in specific tissues, could be increased significantly further.

Plant antibody technology is still in its infancy. However, it offers enormous potential in 'mix-and-match' antibody engineering, and the construction of multimeric immunoglobulin complexes may be feasible relatively easily, for the first time. Furthermore, as there is an enduring interest in using antibodies for therapeutic purposes, agricultural production and distribution offers a means of obtaining large quantities of antibodies at a relatively low cost.

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